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The Evidence of Non *n*-glycan Linked Mannose in Exochitinase 42kDa, from *Trichoderma harzianum* BIO10671 Glycosylation

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ABSTRACT

Chitinase 42 kDa produced by *Trichoderma harzianum* has been proven as a prime compound to be excreted onto the hyphae of the pathogen causing localised cell wall lysis at the point of interaction. Later it will initiate the process of the host cell becomes empty of cytoplasm, disintegrates and shows a rapid collapse. This study investigates the existence of N-glycan linked mannose in chitinase 42 kDa produced by the Malaysian *T. harzianum* strain BIO10671. The chitinase 42 kDa from *T. harzianum* BIO10671 was initially purified using anion exchange chromatography prior to a series of experiments such as immunoblotting against the chitinase 42 kDa antibody, lectin staining for detecting any terminal linked mannose, and galactofuranose detection to determine the presence of galatofuranase components in glycoproteins. The enzyme purification harvested about 12-fold of chitinase 42 kDa from *T. harzianum* BIO10671 with strong indication of the chitinase 42 kDa presence on SDS-Page. This was confirmed by immunoblotting with a strong response around 42 kDa after overnight incubation in chitinase 42 kDa antibody suggesting that the gene for chitinase 42 kDa was greatly expressed in this strain. There are no intervation of galatofuranose on any of the terminal mannose in chitinase 42 kDa as shown by negative results on samples treated with or without endoglycosidase-H and lectin staining. Therefore, it can be concludeed that glycosylation occurred in the chitinase 42 kDa from *T. harzianum* 42 kDa was not in the form of N-glycan linked mannose as expected.

Keywords: *Tricoderma harzianum,* chitinase, glycosylation, N-glycan linked, mannose

INTRODUCTION

The chitinolytic system of *T. harzianum* is complex with several types of chitinase working together simultaneously to fulfil all the niche of *Trichoderma* spp. One of the components in the chitinolytic system is the chitinase 42 kDa which has a molecular weight of around 42 kDa. Filamentous fungi such as *T. harzianum* are able to secrete high levels of protein into the culture medium, and this advantage has been used widely in biotechnology industry to produce heterologous proteins (Archer and Peberdy, 1997). Chitinase 42 kDa by itself is capable of degrading fungal cell walls and its antifungal activity can be enhanced by adding chitinase 33 kDa or combined with gliotoxin (De la Cruz *et al*., 1992; Steyaert *e. al*., 2004). Chitinase 42 kDa from different strains display different physical characteristics such as pI values and substrate specificity (Harman *et al.* 1993; Gakul *et al*., 2000).

Due to its high potential as biocontrol and biotechnology compounds, we are interested to study one of the interesting characteristics of chitinase 42 kDa such as glycosylation, post-translational modification of the protein to strengthen the protein conformation, stability and biological activity in order to produce a high-end protein. One of the problems in producing recombinant glycoprotein is the correct intracellular environment at the moment of glycosylation. Correct intracellular environment may have an effect on the formation on *N*-linked or *O*-linked glycans (Correa, 1982) and both types of glycosylation can effect the function of the final recombinant protein produced (Yan *et al*., 1999). *N*-linked glycan is oligomannose-type glycans that have been modified by the addition of other sugars such as galactose, glucose and galactofuranose, and charged residues such as phosphate and sulphate (Maras *et al*., 1997). Meanwhile, O-linked glycan is mainly composed of short mannose chains close to the C terminus of the protein (Kruszewska *et al*., 1989). However, the glycosylation process is required before glycoprotein production can truly benefit the industry. The aim and objectives of the present study were to obtain the evidence and to confirm the non-N-linked mannose glycosylation in chitinase 42 KDa produced by a Malaysian *T. harzianum* strain BIO10671.

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MATERIALS AND METHODS

Strains

Trichoderma harzianum strain BIO10671 obtained from Department of Biology Culture Collection, Faculty of Science, Universiti Putra Malaysia was used in the present study. The culture was maintained on Potato Dextrose Agar (PDA) at 28^{o}C and subcultured every fortnight.

Extracellular Chitinase Purification

The spore suspensions of *T. harzianum* BIO10671 in approximately $1x10^7$ spores/ml were added to 25 ml of *Trichoderma* Complete Medium (1.0g/l bactopeptone; 0.3g/l urea; 2.0g/l KH_2PO_4; 1.4g/l $MgSO_4.7H_20$; 0.3g/l $CaCl_2.6H_2O$; 0.005g/l $FeSO_4.7H_2O$; 0.002g/l $MnSO_4$; 0.002g/l $ZnSO_4$; 0.002g/l $CoCl_2$; pH 5.5;) supplemented with 0.5% w/v glucose to produce seed cultures. Seed cultures were shaken at 180 rpm at 28^{0}C for 24 h before being filtered through a sterile Whatman No-1 paper. Then were washed three times with sterile distilled water and transferred into 25 ml of *Trichoderma* Minimal Medium (2.0g/l KH_2PO_4; 1.4g/l $MgSO_4.7H_20$; 0.3g/l $CaCl_2.6H_2O$; pH 5.5) supplemented with 1.0% w/v *Pleurotus sajor-caju* mycelium. Three days old culture filtrates were harvested, filtered through a Whatman no.1 filter paper, centrifuged at 6000 x g for 10 min before being dialysed against the distilled water for at least 24h at 4^{0}C.

The purification was carried out using anion-exchange method described by Lima *et al*. (1997). Crude culture containing extracellular chitinase was precipitated with ice-cold 80% acetone and incubated at –20^{0}C for 30min. The precipitate was recovered by centrifugation at 28000 x g for 10 min at 4^{0}C, re-dissolved in distilled water and dialysed against distilled water for another 24 h at 4^{0}C.

Ninety microliter (90 µl) of Buffer A (50mM Tris-HCL pH 7.5) and 20 µl 1M Tris pH 7.5 were added to the dialysed crude culture sample and the pH was adjusted to pH 7.0. The sample was centrifuged at 12000 x g for 10 min. Meanwhile, an anion exchange Neobar AQ column was washed with 10 column volumes of Buffer B (50mM Tris-HCL pH 7.5; 1M NaCl) followed by 10 column volumes of Buffer A. The resulting supernatant was loaded onto the column and eluted at a flow rate of 1ml/min and the bound protein was eluted with a 0-1mM NaCl gradient. The fractions with high β-1,3-glucanase activity were pooled before the dialysis against distilled water for 24 h at 4^{o}C.

Dialysed fraction was collected and assayed for chitinase activity using the chitinase assay method (Reissig *et al*. 1955) which involved the estimation of N-acetlyglucosamine (GlcNAc) released from chito-oligosaccharides by the β-N-acetylhexosaminidase. Discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on collected fraction in 10% acrylamide gels and stained with Coomasie R-250 brilliant blue (Sigma) according to the method of Laemmli (1970). Low molecular mass standard proteins were used for molecular mass determination.

Determination of N-linked glycosylation

Lyophilised sample of purified chitinase 42 kDA was resuspended in 90 µl of nanopure water followed by adding 40 µl 4xbuffer (100mM sodium acetate phosphorus, pH 5.5). The sample was heated for 10 min at 100^{o}C to denature the sample and any contaminating proteases. The sample was then divided into two portions; (i) a control sample i.e. without endoglycosidase-H enzyme (Oxford GlycoSystems) and (ii) test sample with 2µ unit of endoglycosidase-H enzyme added. Finally 5 µl of toluene was added to prevent any fungal or bacteria growth during incubation at 37^{o}C overnight. Four types of assay, namely immunoblotting, lactin staining, galactofuranose detection and general glycan detection were carried out using both of the prepared samples.

Immunoblotting

Treated purified chitinases were tested against chitinase 42 kDA antibody (CHIT42), kindly supplied by Benitez, University of Seville, Spain. Fifty µl lyophilised CHIT42 antibody was diluted 1:2000 in 0.1% (v/v) TBS buffer. This antibody was raised in rabbits against the CHIT42 from *T. longibrachiatum*. Immunoblotting was carried out according to Wallis *et al*. (1999).

Lectin staining: detection of terminal linked mannose

The detection of terminal linked mannose in purified chitinase 42 kDA from *T. harzianum* BIO10671 was done by staining the membrane blots of the chitinase pre- and post- endoglycosidase-H enzyme treatments with *Galanthus nivalis* agglutinin (GNA) lectin (Boehringer Mannheim) according to the manufacturer's recommendation.

Galactofuranose detection

An investigation for the presence of galatofuranose components of glycoproteins was also carried out by blotting against EBA2 antibody kindly provided by Wallis, University of Nottingham, England. The EBA2 is the antibody raised against the immunogenic polysaccharide from *Aspergillus fumigatus,* which recognises glycoproteins containing β-linked galactofuranose residues. The membrane was incubated overnight in EBA2 antibody (2.5 µl in 5ml 0.1% (v/v) TBS-Tween) at 4^{O}C (Wallis *et al*., 1999).

RESULTS AND DISCUSSION

Although chitinase-producing microorganisms are considered an effective biological control, the role of chitinase in the antagonistic process, literatures concerning the purification, molecular properties and molecular structure of extracellular chitinase from mycoparasitic fungi are still limited (Gakul *et al*., 2000; Nompoothiri *et al*., 2004). The crude enzyme from *T. harzianum* BIO10671 contained 26.22 µmoles/µl of chitinase activity was used at the beginning of the anion exchange. The presumed mixture of chitinase was purified from *T. harzianum* using acetone precipitation. The elution pattern of anion exchange chromatography of this crude enzyme fraction is shown in Figure 1 with two peaks for chitinase activities in *T. harzianum* BIO10671

arbitrarily BIO (C1) and BIO (C2) for fraction 4-6 and 24-39, respectively. Only 75.6% (21.33 µmoles/µl) of chitinase activity was recovered after the anion exchange, which consist of 0.18 µmoles/µl in BIO (C1) and 21.15 µmoles/µl in BIO (C2). Since the BIO (C1) contained only a small percentage of chitinase activity; it was not used for further analysis. The analysis of both pooled fractions by SDS-PAGE (Figure 2) showed several major proteins bands in BIO (C1) while only one band was observed in BIO (C2) at approximately 42 kDa.

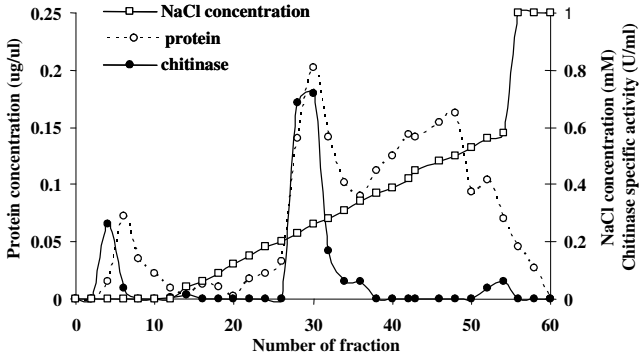

Figure 1: Purification of chitinase by anion exchange chromatography. Bound protein was eluted with a 0 to 0.5M NaCl gradient. Elution profile of *T. harzianum* BIO10671 for protein on Neobar AQ exchanger column with peaks at fraction 4-6 (BIO C1) and fraction 24-39 (BIO C2).

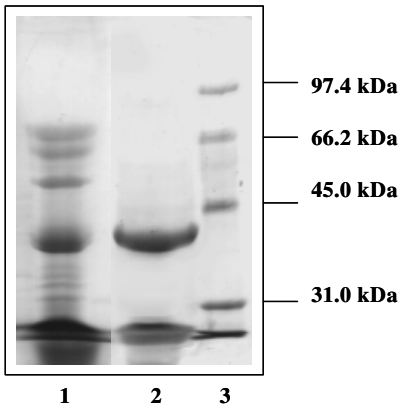

Figure 2: Purification of chitinase by anion exchange chromatography. Bound protein was eluted with a 0 to 0.5M NaCl gradient. SDS-PAGE (10%) of protein from pooled peaks and stained with Coomassie blue.
Note: Lane 1: BIO C1 (Fractions 4-6); Lane 2: BIOC2 (Fractions 24-39); Lane 3: Low range standard Molecular weight Marker.

Recently, several other chitinases have been purified and tested for their antifungal activity (Viterbo *et al.,* 2001; de las Mercedes *et al*., 2001; Harman *et al*., 1993). Three chitinases purified by De la Cruz *et al.* (1992) showed molecular masses of 42, 37 and 33 kDa. The antibodies raised against the two higher molecular mass enzymes reacted specifically and did not cross react suggesting that each protein is encoded by a different gene.

The protein size revealed by SDS-PAGE for *T. harzianum* BIO10671 was 42 kDA which was larger than the purified chitinase collected by Deane *et al.* (1998) from *T. harzianum* T198, but similar to the range of endochitinases reported previously by Ulhoa and Peberdy (1993), Haran *et al*. (1995) and Matsumiya *et al.* (2001). The molecular weight of purified chitinase may differ between species and also within species (Pitson *et al*., 1993) and it is not known whether they are differently processed gene products from the same gene or from separate genes. Sometimes the type of growth substrate used can also influence the number of bands and molecular weight on SDS-PAGE (Vazquez-Garciduenas *et al.,* 1998). Matsuzawa *et al.* (1996) concluded that although the same type of chitinase was purified, characterisation of the purified form will be different from each other and it depends on the species, the type of reaction (exo- or endo-) and the method of purification.

Since *Trichoderma* chitinases are extracellular enzymes, they are expected to be glycosylated. In order to determine whether or not the purified chitinase from *T. harzianum* BIO10671 was *N*-linked glycosylated, a series of tests were carried out including immunoblotting with chitinase 42 kDa antibody, lactin staining, galactofuranose detection, and general glycan detection. Immunoblotting process by incubating the membrane with CHIT42 antibody with the pooled fraction of BIO (C2) revealed four bands within the range of 31 kDA to 45 kDa molecular weight (Figure 3). A strong response around 42 kDa after overnight incubation in CHIT42 antibody suggested that there was a high level of chitinase 42 kDa activity in this strain. In other words, this result indicated that the gene expression for chitinase 42 kDa was greater than the other types of chitinase. This result was parallel to the finding by Carsolio *et al*. (1994) who reported the prominent of chitinase 42 kDa gene expression compared to other genes in the mycolytic strains.

However, another positive reaction was also seen on the membrane from 21 kDa to 97 kDa although only a single chitinase band was detected in SDS-PAGE after the anion purification (Figure 3). This would indicate the existence of chitinase 42 kDa isoform in this strain. The same situation has also been observed by De la Cruz *et al.* (1992) in which three chitinases purified with molecular masses of 42, 37 and 33 kDa did not cross react to the known chitinase antibodies from *T. longibrachiatum* (CHIT42, CHIT37 and CHIT33) , suggesting that each of these proteins was encoded by a different gene. The result is in contrast to the findings reported by Lima *et al.* (1997) who found that only one gene encoded for chitinase 42 kDa in *T. harzianum* T6, and only one band was revealed by the immunoblotting analysis. The differences in type of strain, isoform of enzyme or the possibility of degradation may have influence the

differences in the number of chitinase 42 kDa form. Therefore, further study needs to be carried out to clarify this.

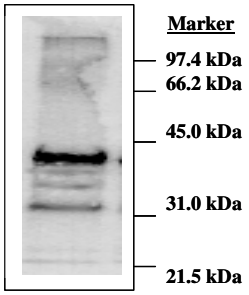

Figure 3: Immunoblot incubated in chitinase 42 kDa antibody (CHIT 42) and detected using secondary anti-rabbit antibody for 1h (1:1000 in 10ml TBS-Tween).
Note: Lane 1:BIO C2 (Fractions 24-39) incubated in 1:2000 CHIT42 dilution in 10 ml 0.1% (v/v) TBS-Tween. Marker: Low range standard molecular weight marker.

Lectin staining using GNA is an effective method to detect any mannose in the glycan component since most glycosylated enzymes in fungi contain mannose (Neethling and Nevalainen, 1995). Prior to this step, purified chitinases from *T. harzianum* BIO10671 was incubated in 37^{o}C in endoglycosidase-H overnight in order to remove any *N*-linked oligosaccharides from the polypeptide so that mannose branches can be easily detected by GNA. There was no positive response for sample treated with or without endoglycosidase-H as shown in Figure 4. This was the first evidence of the non existence of N-glycan link in chitinase 42 kDA. The test also revealed that no lectin stained was aroused at chitinase 42 kDa's position. Thus gave the conclusion of no mannose branches formation in this glycoprotein structure.

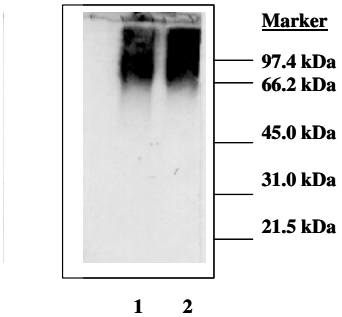

Figure 4: Lectin staining membrane for mannose detection using *Galanthus nivalis* agglutinin (GNA).
Note: Lane 1: BIO C2 (Fractions 24-39) incubated with engoglycosidase-H. Lane 2: BIO C2 (Fractions 24-39) incubated without endoglycosidase se-H. Marker: Low range standard molecular weight marker.

The negative or response to endoglycosidase-H might imply a possibility that the terminal mannose was capped by galatofuranose as these residues were not detected by GNA. To investigate this possibility, a galactofuranose binding antibody was employed. However no galatofuranose could be detected in the chitinase 42 kDa area (Figure 5), thus indicating that galatofuranose was not present in this glycoprotein structure to conceal mannose branches.

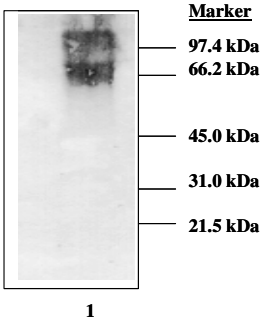

Figure 5: Galactofuranose detection using antibody rose against the immunogenic polysaccharide from *Aspergillus fumigatus* (EBA2) at 4^{0}C.
Note: Lane 1: BIO C2 (Fractions 24-39) against 2.5 µl EBA2 antibody in 5 ml 1% (v/v) TBS-Tween. Marker: Low range standard molecular weight marker.

CONCLUSION

In conclusion, all the results obtained in this experiment through three experiments suggested that chitinase 42 kDa did not exist in the form of *N-glycan* linked mannose glycosylation.

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